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Fibroblast growth factor-2 did not restore plasminogen system activity in endothelial cells on glycated collagen

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ABSTRACT

People with diabetes experience morbidity and mortality from unregulated microvascular remodeling, which may be linked to hyperglycemia. Elevated glucose leads to extracellular matrix collagen glycation, which delays endothelial capillary-like tube formation *in vitro*. Glucose also increases endothelial cell fibroblast growth factor-2 (FGF-2) release and extracellular matrix storage, which should increase tube formation. In this study, we determined if FGF-2 could restore plasminogen system activity and angiogenic function in endothelial cells on glycated collagen. Human umbilical vein endothelial cells cultured on native or glycated collagen substrates were stimulated with FGF-2. Plasminogen system activity, cell migration, and capillary-like tube formation were measured, along with plasminogen system protein and mRNA levels. Glycated collagen decreased endothelial cell plasminogen system activity, cell migration, and tube length. FGF-2 did not restore plasminogen system activity or tube formation in cells on glycated collagen, despite decreasing plasminogen activator inhibitor-1 (PAI-1) protein level. We now show that PAI-1 binds to glycated collagen, which may localize PAI-1 to the extracellular matrix. These data suggest that FGF-2 may not restore angiogenic functions in endothelial cells on glycated collagen due to PAI-1 bound to glycated collagen.

1. Introduction

Diabetic hyperglycemia is implicated in diseases of increased angiogenesis (e.g. diabetic nephropathy and retinopathy) as well as diseases of decreased angiogenesis (e.g., diabetic neuropathy and impaired wound healing) [1–4]. In angiogenesis, endothelial cells must degrade the extracellular matrix so that they can migrate and invade into the surrounding tissue, where they form capillary tubes [5]. Glucose modifies the extracellular matrix in ways that could both increase and decrease angiogenesis. Glucose reacts with amino protein groups to glycate collagen via the Maillard reaction, which enhances collagen cross-linking, makes it more resistant to proteolysis, and inhibits angiogenesis [6–10]. We previously showed that extracellular matrix from endothelial cells cultured in high glucose contains more fibroblast growth factor-2 (FGF-2), and retinal tissue from diabetic patients with proliferative retinopathy showed increased FGF-2 [11,12]. FGF-2 promotes angiogenesis in part by increasing extracellular matrix proteolysis [13].

Extracellular matrix proteolysis in angiogenesis is driven by the plasminogen system and matrix metalloproteinases [14]. In the plasminogen system, urokinase plasminogen activator (uPA) is the primary activator of pericellular matrix proteolysis [15]. Cells secrete uPA as a single chain pro-enzyme (scuPA) [16]. Secreted scuPA binds to its cell surface receptor (uPAR), where plasmin cleaves it to the active two-chain uPA form. uPA then cleaves plasminogen to plasmin, which either directly breaks down matrix components or activates matrix metalloproteinases [17]. Plasminogen activator inhibitor-1 (PAI-1) is the primary uPA inhibitor [18]. Cells secrete PAI-1 in active form but it rapidly decays into its latent form unless it binds to multimeric vitronectin in plasma, platelets, or extracellular matrix [19]. Active PAI-1 binds to and inactivates receptor bound uPA, preventing proteolysis [20]. The uPA:PAI-1 balance is postulated to break down enough of the extracellular matrix to enable cell invasion yet still maintain adequate matrix stability so that cells can adhere to and migrate along the matrix.

Both glycated collagen and FGF-2 impact the plasminogen system, albeit in opposite directions. Chen et al. showed delayed capillary cord branching in human umbilical vein endothelial cells on glycated as compared to native collagen gels, which was ascribed to elevated PAI-1 [10]. Elevated PAI-1 is prevalent in patients with metabolic syndrome, and both diabetic animals and humans showed increased PAI-1 in the arterial wall [21–23]. Thus glycated collagen shifts the plasminogen system balance in the anti-angiogenic direction by increasing PAI-1. FGF-2 increases uPA and PAI-1

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at both the transcriptional and translational levels [24,25]. However, FGF-2 stimulates more uPA than PAI-1. Thus FGF-2 moves the plasminogen system in a pro-angiogenic direction.

In this study, we investigated whether FGF-2 could restore angiogenic functions in endothelial cells on glycated collagen by activating the plasminogen system. Endothelial cells were cultured on native or glycated collagen with or without FGF-2. Plasminogen activity, cell 3D migration, and capillary-like tube formation were measured along with uPA, PAI-1, and uPAR. We now show that FGF-2 did not restore angiogenic functions in cells on glycated collagen despite decreasing PAI-1, perhaps due to increased PAI-1 binding to glycated collagen.

2. Materials and methods

2.1. Cell culture and collagen glycation

Human umbilical vein endothelial cells (HUVEC, passages 4–9; Lonza) were maintained in low glucose (5.5 mM) Endothelium Growth Medium (EGM-2; Lonza) supplemented with 5% fetal bovine serum (FBS; Hyclone), 1% penicillin–streptomycin (Gibco), and 1% glutamine (Gibco). HUVEC were selected because they are widely used for *in vitro* endothelial cell studies, show a robust FGF-2 response, and react with human plasminogen system proteins and antibodies. In addition, HUVEC were used in the original paper in which glycated collagen delayed endothelial cell capillary-like cord branching [10]. Collagen was glycated by incubating 100 μg/mL collagen type I (BD Biosciences) with 500 mM D-glucose-6-phosphate (G6P; Sigma) in phosphate buffered saline (PBS) at 37 °C for 4 weeks. Collagen glycation was validated via autofluorescence and decreased collagenase digestion as described previously [26]. Multimeric vitronectin was created by incubating 1 mg/ml native vitronectin (Molecular Innovations) with 6 M urea (Sigma) in 1 × tris-buffered saline (TBS) for 1 h at 37 °C. After dialysis in 1 × TBS for 18 h, multimeric vitronectin was collected and stored at −80 °C until use. Cell culture substrates were coated with 50 μg/ml native or glycated collagen (BD Biosciences) overnight at 4 °C. After washing, 25,000 cells/cm^2 were seeded on these substrates in Endothelial Basal Medium (EBM-2; Lonza) supplemented with 5% FBS and allowed to attach for 48 h EBM-2, which is EGM-2 without growth factors and cytokines, was used for all experiments to enable measurement of FGF-2 effects.

2.2. uPA activity

Chromozym PL was used to determine uPA activity in cell extracts. In our assay, uPA cleaved exogenous plasminogen to plasmin, which then cleaved Chromozyme PL into a residual peptide and 4-nitroaniline (405 nm). HUVEC were cultured for 48 h on native

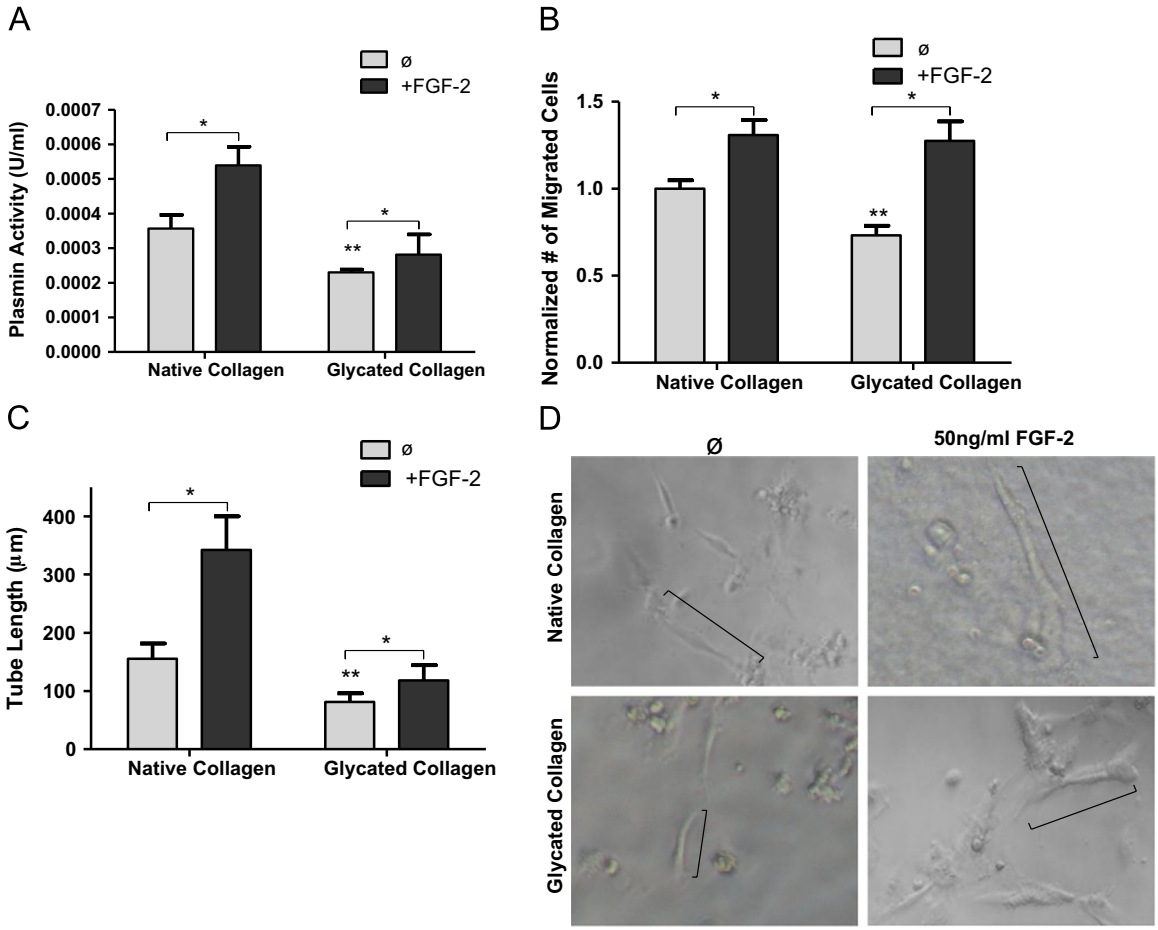

Fig. 1. Glycated collagen decreased endothelial cell uPA activity, 3D migration, and tube length compared to native collagen. FGF-2 only partially abrogated this effect. (A) uPA activity was measured using Chromozym PL. HUVEC seeded on native and glycated collagen coated substrates for 48 h were stimulated with 50 ng/ml FGF-2 for 24 h. (B) 3D cell migration was measured using a Boyden chamber. HUVEC ± 50 ng/ml FGF-2 were added to a Transwell insert coated with 100 μg/ml native or glycated collagen. After 24 h, cells that migrated to the chamber bottom were labeled with Hoechst, imaged by fluorescent microscopy, and quantified with ImageJ. Samples were normalized to native collagen without FGF-2. (C) For tube formation, HUVEC were added to native and glycated collagen gels (4 mg/ml) ± FGF-2 (50 ng/ml). After 18 h, samples were imaged by phase contrast microscopy and tube length was analyzed. (D) Sample tube formation images, with tubes indicated by black arrows. $^{*}p < 0.01$; $^{**}p < 0.01$ glycated vs. native collagen. Each experiment was completed in triplicate and repeated three times.

and glycated collagen coated substrates as described. 50 ng/ml FGF-2 (PeproTech) was added to cells in fresh EBM-2 for 24 h. HUVEC were lysed in T/T buffer (60 mM Tris hydrochloride, 0.5% Triton X-100) for 5 min, after which cell extracts were centrifuged at 10,000 g for 10 min to remove insoluble material. A final solution of cell extract, 127 ng/mL Chromozym PL (Roche), and 67 μU/mL plasminogen (Roche) was mixed in a 96 well plate. Absorbance (405 nm) was measured for 24 h in an Infinite 200 PRO microplate reader (TECAN) maintained at 37 °C. The change in 4-nitroaniline absorbance at 405 nm is directly proportional to uPA enzymatic activity. Absorbance was plotted vs. time, and the linear region slope (ΔA/min) was used to calculate uPA activity via the following equation:

$$\text{uPA Activity}\,(\text{U/ml}) = [(V/v\cdot\epsilon\cdot d)\cdot(\Delta A/\text{min})].$$

where V is total volume (300 μl), v is cell extract volume (33 μl), ϵ is absorbance coefficient for 4-nitroaniline ($1.4\times10^4\ \text{mol}^{-1}\ \text{cm}^{-1}$), and d is light path (1 cm).

3. 3D migration

HUVEC 3D migration was assessed using a Boyden chamber assay. The bottom of a Transwell chamber (6.5 mm diameter, 8 μm pore size; Corning Costar) was coated with 100 μg/ml native or glycated collagen, which was allowed to gel for 1 h at room temperature. 150,000 cells were then added to the top of each chamber in EBM-2 with 5% FBS with and without FGF-2 (50 ng/ml). EBM-2 with 10% FBS was added to the bottom chamber. Samples were incubated for 24 h at 37 °C, after which cells remaining in the upper Transwell chamber were removed by swabbing. Cells that migrated to the chamber bottom were labeled with Hoechst (10 ng/ml; Invitrogen) for 30 min. 5 randomly selected areas per sample were imaged using an Olympus IX81 inverted fluorescent microscope. Cell number was quantified with ImageJ.

3.1. Tube formation

HUVEC tube formation was assessed on native or glycated collagen gels. Native collagen gels were prepared by incubating 50 μl 4 mg/ml collagen I at 37 °C for 1 h. Glycated collagen gels were prepared by incubating native collagen gels with 500 mM G6P for 4 weeks at 37 °C. 15,000 HUVEC were added to each gel in EBM-2 with 5% FBS with and without FGF-2 (50 ng/ml). Cells were incubated for 18 h at 37 °C, after which samples were imaged using a Nikon Eclipse TS100 phase contrast microscope (5 images per well). Tube length was manually analyzed using ImageJ by an objective technician with no knowledge of the coded images [27].

3.2. uPA, PAI-1, and uPAR protein and mRNA levels

uPA, PAI-1, and uPAR protein and mRNA levels were quantified by Western blot and real-time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR), respectively. HUVEC were cultured for 48 h on native or glycated collagen coated substrates as described, after which they were treated with 50 ng/ml FGF-2 for 2–24 h. For Western blot, cells were scraped off the surface in ice-cold lysis buffer (20 mM Tris, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 2 mM EDTA, 2 mM $NaVO_4$, 2 mM PMSF, 50 mM NaF, 10%

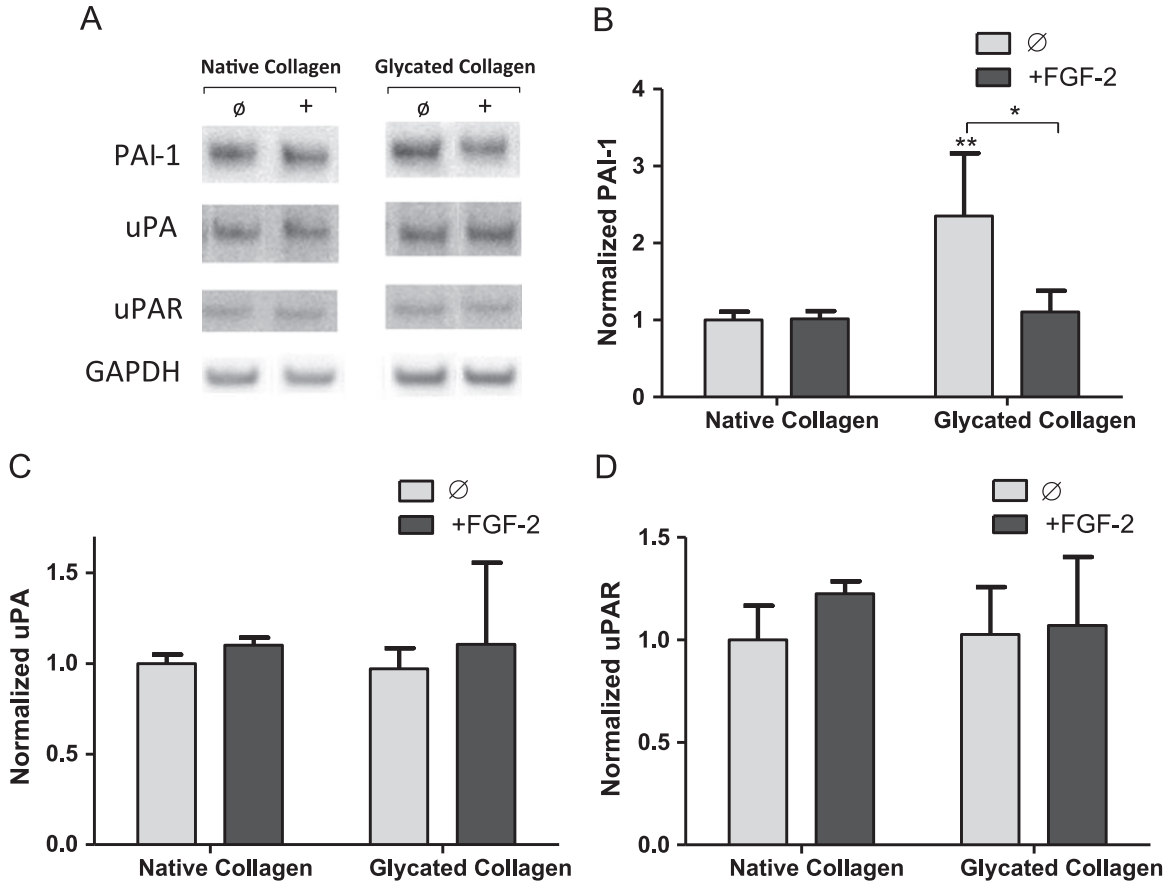

Fig. 2. Glycated collagen increased PAI-1 protein level, and FGF-2 treatment returned PAI-1 back to the native collagen level at 24 h. (A) HUVEC seeded on native and glycated collagen coated substrates for 48 h were stimulated with 50 ng/ml FGF-2 for 24 h. Cell extracts were collected and normalized protein samples were analyzed by Western blot. (B, C, D) Band intensity for PAI-1, uPA, and uPAR, respectively, was quantified and normalized to GAPDH. 1 representative Western blot of 3, with 3 samples each. $^{*}p<0.01$; $^{**}p<0.01$ glycated vs. native collagen. Each experiment was completed in triplicate and repeated three times.

glycerol, complete protease inhibitor, pH 7.4). Cell lysates were normalized for protein content, separated by SDS-PAGE on a 4–12% Bis-Tris gel (Invitrogen), and transferred to nitrocellulose membranes using the Invitrogen i-Blot system. Membranes were incubated overnight at 4 °C with primary antibodies to uPA, PAI-1, or uPAR (Santa Cruz), followed by a secondary horseradish peroxidase-conjugated antibody (Promega) for 2 h at room temperature. Protein bands were detected using an enhanced chemiluminescence kit (Western Lightning, PerkinElmer) and visualized with a Fluorchem digital imager (Alpha Innotech). Band intensity was quantified using AlphaEase FC software.

For RT-PCR, RNA was isolated using an RNeasy Mini Kit (Qiagen) according to the manufacturer protocol. RNA concentration was quantified by measuring sample absorbance (260 nm) using a NanoDrop 1000 (Thermo Scientific), and RNA purity (1.8–1.99 for all samples) was determined using the 260/280 nm ratio. RNA samples were stored at −80 °C until use. Primers for uPA (forward: 5′-AATGACTGTTGTGAAGCTGATTTC-3′; reverse: 5′-AGGGGTCCCCCTGAGTCT-3′), PAI-1 (forward: 5′-TCCAGCAGCTGAATTCCTG-3′; reverse: 5′-GCTGGAGACATCTGCATCCT-3′), and GAPDH (forward: 5′-ACCACAGTCCATGCCATCAC-3′; reverse: 5′-TCCACCACCCTGTTGCTGTA-3′) were from Integrated DNA Technologies. GAPDH was selected as the housekeeping gene because its mRNA levels do not change in HUVEC treated with FGF-2 [28]. PCR was performed on a LightCycler 480 Real-Time PCR System using LightCycler 480 RNA-Master Hydrolysis probes (Roche) according to the manufacturer protocol. Each 20 µl reaction contained 150 ng total RNA, 0.4 µM Universal Probe #66 (Roche), 0.5 µM uPA or PAI-1 primer, 3.25 mM $Mn(OAc)_2$, 1 × Master Hydrolysis Probes and 1 × Enhancer (Roche). Thermal cycling conditions were set as follows: 1 cycle reverse transcription at 63 °C for 20 min; 1 cycle denaturation at 95 °C for 30 s; and 45 cycles amplification (as per manufacturer instructions) and annealing at 95 °C for 10 s with a second amplification at 60 °C for 45 s with single acquisition mode. The protocol was selected to allow primers to anneal at a greater efficiency. In all cases, the Ct values were between 17 and 25. mRNA level was quantified using both the standard curve and the second derivative maximum method. All standard curves and samples were determined to have a maximum error of 0.05 and doubling rate greater than 1.85. All reactions were performed in triplicate. Reactions without the template DNA (no template controls, NTC) and without cell sample were the negative controls.

3.3. PAI-1 binding to extracellular matrix proteins

PAI-1 binding to native collagen, glycated collagen and vitronectin was assessed using a solid phase binding assay [29]. 96 well plates were coated with the appropriate extracellular matrix protein as described and blocked using PBS with 3% BSA and 0.05% Tween. 1 µg/ml PAI-1 (in tris-buffered saline with 1% BSA, 0.01% Tween, and 1 mM $CaCl_2$) was added to each well and incubated for 2 h at 37 °C. After thorough washing, bound PAI-1 was detected by incubating samples with a PAI-1 primary antibody (Santa Cruz) followed by a horse radish peroxidase-conjugated secondary antibody (Promega). The assay was developed using a two-step process to decrease background absorbance. First, 1.8 nM 2,2-azinobis(3-ethylbenzthiazidine-6-sulfonic) acid (Sigma) in 0.1 M sodium citrate, pH 4.5 was added to samples for 30 min at 37 °C. This

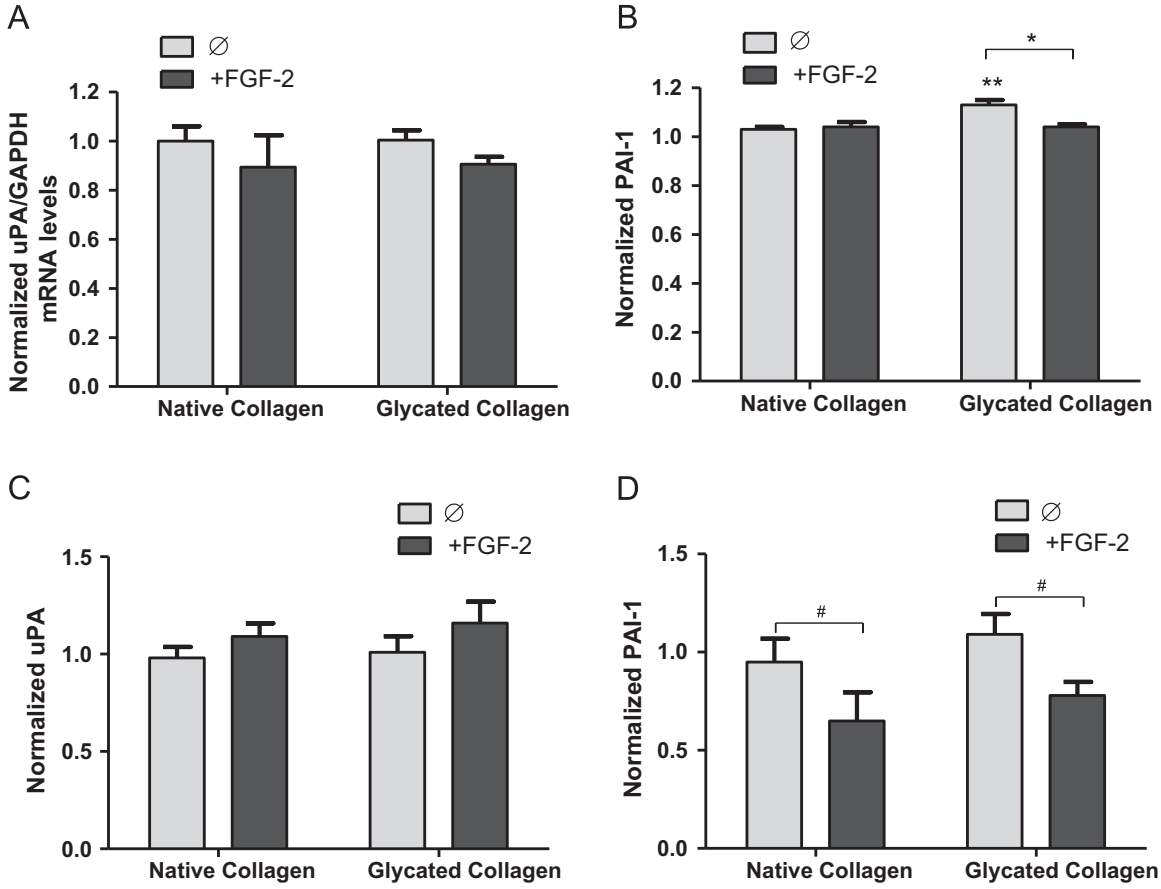

Fig. 3. Glycated collagen did not alter uPA or PAI-1 mRNA, whereas FGF-2 treatment decreased PAI-1 mRNA on both native and glycated collagen. HUVEC seeded on native and glycated collagen coated substrates for 48 h were stimulated with 50 ng/ml FGF-2 for (A, B) 2 or (C, D) 24 h. One-step RT-PCR was performed using a LightCycler 480. (A, C) uPA and (B, D) PAI-1 mRNA levels were normalized to GAPDH for each condition. $^{\#}p < 0.05$, $^{*}p < 0.01$, $^{**}p < 0.01$. Each experiment was completed in triplicate and repeated three times.

was then replaced with 1.8 mM 2,2-azinobis(3-ethylbenzthiazidine-6-sulfonic) acid and incubated for 30 min at 37 °C. Absorbance (405 nm) was quantified using a microplate reader.

3.4. Statistical analysis

Statistical analysis was performed with GraphPad Prism and Instat software. Samples were collected in triplicate and experiments performed at least two times. Data are graphed as mean ± standard deviation. Significance between two groups was compared using Student's *t*-test. Comparisons among multiple groups were analyzed by two-way ANOVA with a Bonferroni post-hoc test. *p*-values are indicated in the figures by $^{\#}p < 0.05$, $^{*}p < 0.01$, $^{**}p < 0.001$, unless otherwise indicated.

4. Results and discussion

4.1. Endothelial cell response to FGF-2 on native and glycated collagen

Glycated collagen was previously shown to delay endothelial cell capillary cord branching, perhaps due to elevated PAI-1 [10]. We therefore measured whether FGF-2 could reverse glycated collagen-induced changes in plasminogen activity and angiogenic functions. HUVEC attachment and spreading were statistically similar on both native and glycated collagen coated substrates (data not shown). However, uPA activity was 35% lower in cells on glycated compared to native collagen (Fig. 1A). While FGF-2 increased uPA activity by 51% in cells on native collagen, it only increased uPA activity by 22% in cells on glycated collagen. Cell proliferation (assessed by cell counts) and 2D migration (assessed by cage assay) increased with FGF-2 on both native and glycated collagen, with no differences based on substrate composition (data not shown). In contrast, collagen glycation did change endothelial cell 3D migration through a collagen coated Boyden chamber. 27% fewer cells migrated through the Boyden chamber on glycated as compared to native collagen. FGF-2 stimulation restored cell migration on glycated collagen back to native collagen levels (Fig. 1B). Endothelial cell tube length was 48% lower for HUVEC on glycated collagen gels as compared to native collagen gels. FGF-2 more than doubled tube length on native collagen gels, but only increased tube length 46% on glycated collagen gels (Fig. 1C; sample images in Fig. 1D). Thus FGF-2 fully rescued 3D migration but not plasminogen system activity or capillary-like tube formation from glycated collagen effects.

4.2. Glycated collagen and FGF-2 effects on plasminogen system components

Plasminogen system activity derives from uPA binding to uPAR and the uPA:PAI-1 balance. We therefore measured uPA, uPAR, and PAI-1 protein levels by Western blot (Fig. 2A) to determine which proteins contributed to decreased plasminogen system activity in cells on glycated collagen. For the selected time points (48 h on glycated collagen followed by 2, 6, 12, or 24 h of FGF-2 stimulation), glycated collagen and FGF-2 only changed PAI-1 protein levels at 24 h. HUVEC on glycated collagen showed more than double the PAI-1 protein as cells on native collagen, and FGF-2 brought the PAI-1 level down to that of cells on native collagen (24 h data in Fig. 2B). FGF-2 had no effect on PAI-1 protein in cells on native collagen, and neither glycated collagen nor FGF-2 affected uPA or uPAR levels across all time points (24 h data in Fig. 2C and D).

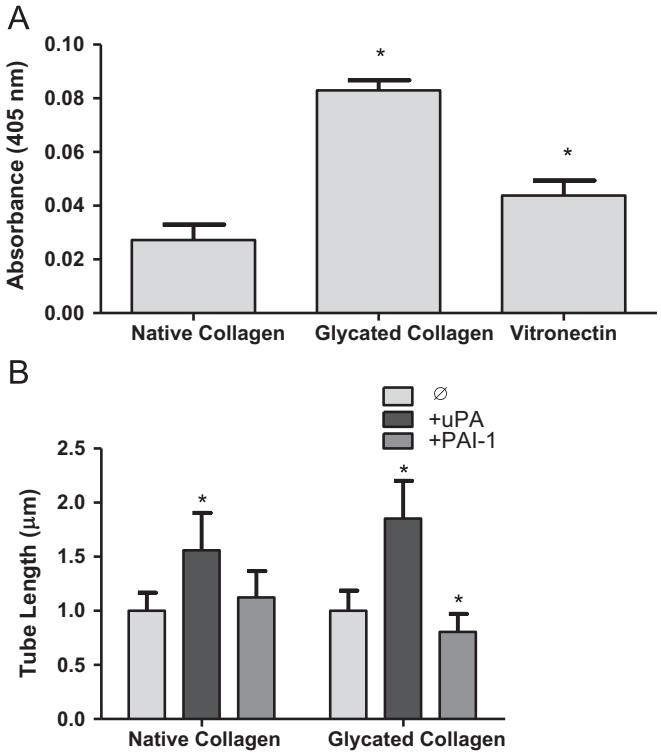

Fig. 4. PAI-1 bound to glycated collagen more than to native collagen, and exogenous PAI-1 only decreased tube length in endothelial cells on glycated collagen. (A) Plates coated with native collagen, glycated collagen, or multimeric vitronectin were incubated with 1 μg/ml PAI-1. A solid-phase binding assay was used to detect bound PAI-1. $^{*}p < 0.01$ compared to native collagen. (B) 15,000 HUVEC were added to native or glycated collagen gels ± 12 IU/ml uPA or 1 ng/ml PAI-1. After 18 h, tubes were imaged by phase contrast microscopy and tube length was analyzed with ImageJ. Samples were normalized to the control without uPA or PAI-1 on the respective substrate to best compare uPA and PAI-1 effects. $^{*}p < 0.01$ compared to samples without uPA or PAI-1 on the respective substrate. Each experiment was completed in triplicate and repeated at least two times.

We next measured uPA and PAI-1 mRNA levels at the same time points to determine if changes in PAI-1 protein levels were related to PAI-1 production. Neither glycated collagen nor FGF-2 affected uPA mRNA at 2 h, which was consistent with Western blot data (Fig. 3A). Glycated collagen induced a small but significant increase in PAI-1 mRNA at 2 h, and FGF-2 decreased this effect (Fig. 3B). After 24 h, uPA mRNA was similar across all samples but FGF-2 decreased PAI-1 mRNA by 30% in cells on both native and glycated collagen (Fig. 3C and D).

4.3. PAI-1 binding to glycated collagen

We and others previously showed that matrix protein denaturation and glycation can induce structural changes that alter cell and protein binding [30–33]. We therefore hypothesized that PAI-1 levels increased in cells on glycated collagen partially due to PAI-1 binding. Indeed, more than three times as much PAI-1 bound to glycated collagen as compared to native collagen in an equilibrium binding assay (Fig. 4). FGF-2 did not displace the PAI-1 bound to glycated collagen (data not shown). We then added exogenous uPA and PAI-1 to HUVEC cultured on native or glycated collagen gels. Whereas uPA increased capillary-like tube length on both substrates, PAI-1 only decreased tube length in cell on glycated collagen gels. These data suggest that PAI-1 binding to glycated collagen mediates its effect on angiogenic processes.

Glucose induced changes in the extracellular matrix, such as collagen glycation, decrease endothelial capillary-like tube formation *in vitro* [10]. We previously showed that glucose also increases endothelial FGF-2 release and matrix storage [11]. We now show that FGF-2 only partially restored plasminogen system activity in cells on glycated collagen. FGF-2 decreased PAI-1 levels in cells on glycated collagen. However, FGF-2 did not decrease PAI-1 binding to glycated collagen, which may localize PAI-1 to the extracellular matrix [30,31,34,35]. While PAI-1 binding and activity were previously shown to increase with glycated fibrin [32], we are the first to report that PAI-1 also binds to glycated collagen, and that this may modulate plasminogen system activity.

While these studies describe interesting new interactions among growth factors, glycated collagen, and the plasminogen system, they are not without limitations. We used HUVEC to relate our results to previous work in the literature and because they form capillary-like tubes *in vitro*. However, microvascular endothelial cells may be a better model for future work. In our experiments, FGF-2 did not affect uPA or PAI-1 mRNA. This was initially surprising, since others had previously shown that FGF-2 regulates both uPA and PAI-1 [24,25]. However, FGF-2 typically has less effect on the plasminogen system in human as compared to animal cells, and therefore continuous FGF-2 stimulation or mediator proteins may be required to enhance FGF-2 effects [14,24,36]. In fact, when we added fibrinogen to our FGF-2 stimulated samples, we did observe significant increases in uPA and PAI-1 mRNA. Finally, some of our observed effects on plasminogen system activity could relate to tissue plasminogen activator (tPA), rather than uPA. tPA is produced by endothelial cells, although it is primarily thought to generate plasmin for blood fibrinolysis [37]. We focused on uPA due to its importance in angiogenic functions; however the possibility of tPA effects cannot be excluded.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bbrep.2015.09.001.

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